Spring 5-5-1965

Effect of 6-Mercaptopurine on Glucose Disappearance and Lactate Formation in the Media of Resistant and Sensitive Human Neoplastic Cells in Vitro

Richard John Kraemer
University of Colorado Boulder

EFFECT OF 6-MERCAPTOPURINE ON GLUCOSE DISAPPEARANCE AND LACTATE FORMATION IN THE MEDIA OF RESISTANT AND SENSITIVE HUMAN NEOPLASTIC CELLS IN VITRO

by

Richard John Kraemer

B.S., University of Wisconsin, 1962

A Thesis submitted to the Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirements for the Degree

Master of Sciences

Department of Pharmacology

1965

This Thesis for the M.S. degree by

Richard John Kraemer

has been approved for the

Department of

Pharmacology

by

Richard A Whitehead

Richard A. Deitrick

Date May 10, 1965

Acknowledgments

I wish to express my sincere gratitude and appreciation to Dr. Richard A. Deitrich and Dr. Richard W. Whitehead under whose direction this research was conducted.

I also wish to thank Mr. Clifford Meyers and Mrs. Ailene Hofer for their aid in maintaining the cell lines.

I am indebted to Miss Joan Davies for her secretarial assistance.

This research was supported in part by United States Public Health Service Grant number CA-06231.

Kraemer, Richard John (M.S., Pharmacology)

Effect of 6-Mercaptopurine on Glucose Disappearance and Lactate Formation in the Media of Resistant and Sensitive Human Neoplastic Cells in Vitro

Thesis directed by Professor Emeritus Richard W. Whitehead

Human neoplastic cells resistant to the antileukemic agent 6-mercaptopurine (6-MP) were examined in tissue culture to study the glycolytic activity of resistant cells. The media, with and without 6-MP, of three neoplastic cell lines, HeLa, HEp-2, and J-111 was compared for glucose and lactate concentrations before and after incubation. It was found that in the media of HeLa and HEp-2 cells exposed to 6-MP glucose disappearance and lactate formation was enhanced. It was also found, however, that glucose and lactate concentrations of the media of 6-MP resistant HeLa and HEp-2 cells were not altered by the activity of 6-MP. On the other hand, in the media of J-111 cells both sensitive and resistant to 6-MP glucose and lactate concentrations were not changed by the activity of 6-MP.

The results indicate that with the development of resistance to 6-MP, HeLa and HEp-2 cells no longer are stimulated by 6-MP to use extracellular glucose at an increased rate. It is also known that when HeLa and HEp-2 cells develop resistance to 6-MP there is an associated inability of both cell types to form the 6-MP ribonucleotide. The interrelationships of these two findings is not known; although it is suggested that both cell types develop resistance by a common pathway. However, because glycolytic activity in J-111 cells is not stimulated by 6-MP it is suggested that resistance may develop by still another mechanism.

This abstract of about 250 words is approved as to form and content.
I recommend its publication.

Signed Richard J. Whitehead
Instructor in charge of dissertation

Table of Contents

Introduction

The most recent mortality statistics available reveal cancer as the second leading cause of death in the United States; cardiovascular diseases rank first.(32) Two thirds of the patients with diagnosed malignancies do not survive five years.(33) Surgery and radiation therapy are presently the chief measures of cancer control. Within the last 15 years, however, temporary control of certain cancers has been achieved by the use of chemical agents; e.g., the treatment of acute leukemia with 6-mercaptopurine (6-MP). Although the chemotherapeutic approach to cancer has met with moderate success, a major stumbling-block plagues advancement. The development of drug resistance[1] remains a serious obstacle in the progress of cancer chemotherapy. Considerable effort has been made at the molecular level to gain knowledge regarding the nature of the altered metabolism within the drug-sensitive cells with the hope that such knowledge might facilitate an understanding of drug resistance.

The purine analog, 6-MP, has been closely examined for its chemotherapeutic activity and it has been found that 6-MP is capable of altering many metabolic processes and cell functions within 6-MP-sensitive cells. Some of the metabolic pathways and cell functions found to be sensitive to the activity of 6-MP include: the de novo synthesis of purines (3-6,12,13,23); the formation of antibodies (14,27,28); the synthesis and breakdown of NAD (1,2,17); and the utilization of glucose. (8,9,10,15,18,19,20,22) These studies have revealed a primary site of

---

[1] The chemical selection and overgrowth of specific drug-resistant mutant cells from large, originally drug sensitive populations of neoplastic cells.

the growth-inhibitory activity of 6-MP. It is now generally considered that the de novo synthesis of purines is the pathway most sensitive to the inhibitory activity of 6-MP.

Because the de novo synthesis of purines appears to be a sensitive site of the inhibitory activity of 6-MP, the study of 6-MP resistance has focused on this mechanism. It has been reported that 6-MP must be converted to 6-MP ribonucleotide in sensitive cells to be an inhibitor of the de novo synthesis of purines.(5,6,12,23) The 6-MP ribonucleotide has been found to be a potent inhibitor of the first irreversible step of purine synthesis (the free base and 6-MP ribonucleoside were inactive).(21) Studies on resistance have revealed that in some tumor cells examined 6-MP resistance is accompanied by the inability of the cells to form the 6-MP ribonucleotide.(5,6,23) However, it has not always been possible to demonstrate the inability of 6-MP-resistant cells to form the 6-MP ribonucleotide.(5,7) Obviously our knowledge of the mechanism of resistance still has many gaps.

It was felt, therefore, that the understanding of 6-MP resistance might be enhanced by an examination of the glycolytic activity of 6-MP resistant cells. The activity of 6-MP on aerobic and anaerobic glycolysis (the metabolism of glucose to lactate in the presence and absence of oxygen) has been studied in a variety of sensitive tumor cells. These studies, outlined below, provide a basis from which to study the glycolytic activity of 6-MP in resistant cells.

The action of 6-MP on aerobic and anaerobic glycolysis has been examined in a number of tumor preparations. In the majority of tumor cells examined, 6-MP appears to enhance aerobic glycolysis, but does not affect anaerobic glycolysis. Ebina et al. measured anaerobic gly-

colysis in 6-MP treated Yoshida ascites sarcoma, Ehrlich ascites carcinoma, and rat ascites hepatoma cells.(8) They showed that 6-MP had no significant influence on anaerobic glycolysis. The effect of 6-MP on anaerobic glycolysis was determined in human acute lymphatic and acute myelogenous leukemic cells.(20) It was found that 6-MP did not alter anaerobic glycolysis in acute leukemic cells. The action of 6-MP on anaerobic glycolysis in cell free systems has also been examined and the findings confirm those of Ebina et al. (8) and Laszlo et al.(20) Hochstein prepared an actively glycolyzing supernatant fraction from S91 mouse melanoma.(16) He showed that 6-MP had no measurable effect on anaerobic glycolysis in the supernatant fraction; anaerobic glycolysis in the mitochondrial fraction treated with 6-MP was inhibited 10-25%.(17)

It appears that 6-MP has no effect on anaerobic glycolysis in those tissues examined with one exception: anaerobic glycolysis in the Crocker mouse sarcoma 180 tumor appears to be uniquely sensitive to 6-MP. Mihich et al. reported a 50% inhibition of anaerobic glycolysis in Crocker sarcoma 180, carried in 6-MP treated mice.(22) Fodor et al. have revealed several enzymatic sites of 6-MP inhibition of anaerobic glycolysis in sarcoma 180 homogenates.(9,10) Fodor's findings, however, have not been confirmed by reports from other investigators using different tumor cell preparations. It therefore appears that the glycolytic inhibitory activity of 6-MP is peculiar to the Crocker mouse sarcoma 180 tumor.

The action of 6-MP on aerobic glycolysis has also been examined. Laszlo et al. reported that 6-MP had no effect on aerobic glycolysis in acute myelogenous leukemic cells, although aerobic glycolysis was

slightly inhibited in acute lymphatic leukemic cells.(20) Ebina et al. showed that in cells from three different types of rat ascites tumors examined, 6-MP did not significantly alter aerobic glycolysis.(8) Ebina's findings have been recently confirmed in many types of human leukemic cells and ascites cells.(18,19) Katchman et al. showed that 6-MP increased aerobic glycolysis in their tumor cell preparations; however, the activation of aerobic glycolysis by 6-MP was found to be dependent on the concentration of 6-MP employed.(19) In their experiments in which human leukemic leukocytes and human ascites cells were used, 6-MP concentrations of 2-50 $\mu g/10^6$ cells stimulated aerobic glycolysis (as measured by lactic acid production), whereas 6-MP concentrations above 50 $\mu g/10^6$ cells inhibited aerobic glycolysis.(19) These findings suggest that at low concentrations, i.e., those concentrations which may be achieved under normal physiological conditions, 6-MP stimulates aerobic glycolysis.

To our knowledge alterations of glucose utilization have not been examined as a possible cause of development of 6-MP resistance. In view of the evidence cited, it was felt that an examination of the glycolytic activity of resistant cells might contribute more knowledge to the understanding of 6-MP resistance. In the following study, it was our purpose to evaluate the relative effects of 6-MP on glucose uptake and lactate formation by 6-MP-resistant human cancer cells grown in cell culture.

Materials and Methods

Human tumor cells cultivated in tissue culture offer the advantage of relative uniformity, controlled growth conditions, and suspensions which are especially suitable for metabolic studies. Of the three serially propagated human cell lines used in the following experiments, one was derived from cervical epithelioid carcinoma (HeLa) (11), one from laryngeal epidermoid carcinoma (HEp-2) (31) and one from acute monocytic leukemia (J-111) (25).

The initial culture of HeLa cells was obtained from Dr. T. T. Puck. HEp-2 and J-111 cells were purchased from Baltimore Biological Laboratories, Baltimore, Maryland. All cell lines and sublines were grown attached to soft glass 60 mm Petri dishes, unless otherwise stated. The basic medium used in all cultures was composed of 42.5% synthetic nutrient (N-16) (26), 42.5% balanced salt solution (Saline F) (26) and 15% calf serum. The drug medium was identical to the basic medium with the addition of 6-MP. Media were changed every three days and all cell lines and sublines were subcultured every six days.

Incubation was carried out at 37° in a tight box continuously flushed with a water-saturated mixture of 5% CO_2 in air. The humidity of the box was kept at 90%. The culture media were maintained at pH 7.3. Modal chromosomal range for each cell line was: HeLa 58-86, HEp-2 63-81 and J-111 149-188. A count of the per cent polynucleated cells was determined and it was found that the cell lines contained 10-15% and the sublines 15-20% polynucleates.

Resistant sublines of HeLa cells were developed to: 1.0, 1.5 and

2.0 µg/ml of 6-MP, respectively. HEp-2 and J-111 cell sublines were made resistant to 1.0 µg/ml. The 6-MP resistant cells were produced by seeding Petri dishes with 2 x 10^5 cells suspended in drug medium. The media were changed twice weekly and the non-attached cells were drawn off. Those cells that remained viable after four months continuous exposure to 6-MP were considered drug-resistant.

The cell samples were prepared in the following manner: 4 to 5 large 60 mm glass Petri dishes were each seeded with 2 x 10^5 cells suspended in 3 ml of the desired medium and allowed to incubate 3 days. The cells were then detached from the glass by incubation for 5 minutes in 0.01% sodium ethylenediaminetetraacetate and 0.025% trypsin solution. The cells from all of the glass dishes were combined into a single tube and centrifuged 5 minutes at 250 x G. The packed cells were then: washed with a balanced salt solution (Saline F); resuspended in the desired medium, and counted in a Coulter Counter using a 100 µ aperture with threshold voltage and aperture current adjusted to count only those particles larger than 28 µ in diameter. The final cell samples were drawn from this inoculum.

To 35 mm plastic culture dishes were added 2 x 10^6 cells suspended in 1 ml of medium. The cell suspension media, devoid of cells, were prepared in a similar manner to determine initial concentrations of glucose and lactic acid. All samples were then incubated 4 hours, at which time the media were immediately withdrawn and centrifuged (10 minutes at 2000 x G) under refrigeration to remove unattached cells. Approximately 5% of the original inoculum did not attach to the plastic culture dishes. All media were then analyzed quantitatively for glucose and lactic acid.

The analyses for glucose were performed using the method described

by O'Brien and Ibbott.(24) In this method glucose is specifically estimated by using the anzyme glucose oxidase. This enzyme reacts with its substrate D-glucose, to give D-gluconic acid and hydrogen peroxide. The reaction of hydrogen peroxide with a receptor, O-tolidine, is catalyzed by peroxidase to give a blue color bearing a linear relationship to the concentration of glucose present. The optical density of the chromatic solution is read in the Beckman DU spectrophotometer at 625 mμ.

Lactic acid was assayed using a commercial preparation of lactate dehydrogenase (Calbiochem, Inc.). Known concentrations of calcium-L-lactate (1 and 5 μ moles lactate/ml) were compared routinely with each sample. In the presence of excess NAD, lactate is converted to pyruvate, which is trapped as the hydrazone, with NAD being stoichiometrically reduced to NADH. The optical density of the NADH is read at 340 mμ in the Beckman DU spectrophotometer.

Results

Effect of various 6-MP concentrations on glucose disappearance from the culture medium of drug-sensitive and drug-resistant HeLa cells.

In these experiments the influence of various concentrations of 6-MP on glucose disappearance was examined. It was found (Table I) that the amount of glucose which disappeared from the medium of drug-sensitive HeLa cells was independent of the 6-MP concentrations (1.0, 1.5, 2.0 µg/ml) added to the medium. The amount of glucose which disappeared from the medium of drug-sensitive cells exposed to 6-MP was twice as great as the quantity of glucose disappearing from the medium of untreated, drug-sensitive cells. When both sensitive and resistant cells were exposed to identical concentrations of 6-MP, the medium of the resistant cells contained one half the amount of glucose found in the medium of the sensitive cells. Although the three cell sublines were resistant to three different concentrations of 6-MP, they showed no differences in glucose disappearance. The media of resistant and untreated sensitive HeLa cells displayed comparable glucose concentrations after incubation.

Effect of 6-MP on glucose disappearance and lactate formation in the media of HeLa, HEp-2, and J-111 cells sensitive or resistant to 6-MP.

The three cell lines were compared as to their aerobic glycolytic activity (as measured by the media lactate concentration) before and after treatment with 1.0 µg/ml of 6-MP. The data in Table II reveal that lactate concentrations were greater in the media of 6-MP treated sensitive HeLa and HEp-2 cells than in the media of the untreated cells. Glucose disappearance was also enhanced when sensitive HeLa

and HEp-2 cells were treated with 6-MP. Glucose disappearance and lactate formation were quantitatively unaffected in the media of 6-MP treated and untreated sensitive J-111 cells.

The three resistant cell sublines were compared to their corresponding sensitive cell lines for glycolytic activity in the presence of 6-MP (Table III). It was found that the media of resistant HeLa and HEp-2 cells contained less lactate than the media of the 6-MP treated, sensitive cells. The media of resistant HeLa and HEp-2 cells also exhibited less glucose disappearance than the treated, sensitive cells media. The level of lactate in the media of resistant HeLa and HEp-2 cells was comparable to the lactate concentration in the media of the untreated sensitive cells. The media of resistant and sensitive 6-MP treated and untreated J-111 cells were found to contain equivalent concentrations of lactate. All resistant cell sublines were indistinguishable from their corresponding untreated cell lines as measured by glucose disappearance and lactate formation in the incubation media.

The quantitative levels of aerobic glycolysis in the three untreated cell lines were found to be in the following descending order: J-111> HEp-2> HeLa. The development of resistance to 6-MP in the cell sublines did not alter the order of glycolytic activity seen in the cell lines. However, the glycolytic order of activity of the 6-MP treated sensitive cell lines was: J-111> HeLa> HEp-2.

Table I

Each culture dish was inoculated with 2 x 10^6 cells suspended in 1 ml of basic or drug medium.

Glucose disappearance is the difference between the glucose concentration at time zero and the glucose concentration in the dish after 4 hours of incubation. Each figure represents an average of the indicated number of determinations. Glucose disappearance values were compared for significance using the Student "t" Test. All values were considered significant at P = .02.

Table I

Glucose disappearance from the culture media of HeLa cells sensitive and resistant to various concentrations of 6-MP.

6-MP (micrograms/ml)	Glucose Disappearance (micromoles/ml) Sensitive	Resistant	P*
0	1.32 ± .14† (9)§	——	
1.0	3.16 ± .16 (10)	1.64 ± .36 (10)	.001
1.5	3.25 ± .13 (12)	1.41 ± .27 (10)	.001
2.0	3.15 ± .14 (9)	1.62 ± .22 (9)	.001

* Probability (Student "t" Test)

† Standard Deviation. S.D. $= \sqrt{\frac{\Sigma(X-\overline{X})^2}{n-1}}$

§ Number of determinations.

Table II

Each culture dish was inoculated with 2×10^6 cells suspended in 1 ml of basic or drug medium.

Glucose disappearance is the difference between the glucose concentration at time zero and the glucose concentration in the dish after 4 hours of incubation. Lactate formed is the net increase in lactic acid found in the media after incubation. All figures represent averages of the indicated number of determinations. All values were analyzed for significance using the Student "t" Test and were considered significant at $P = .02$.

Table II

The effect of 6-MP on glucose disappearance and lactate formation in the media of HeLa, HEp-2, and J-111 cells sensitive to 6-MP.

	Glucose Disappearance (micromoles/ml)		P*	Lactate Formation (micromoles/ml)		P*
	Sensitive	Sensitive + 6-MP		Sensitive	Sensitive + 6-MP	
HeLa	1.60 ± .12† (9)§	3.23 ± .44 (10)	.001	1.45 ± .14 (9)	2.87 ± .16 (10)	.001
HEp-2	2.15 ± .07 (9)	2.52 ± .17 (12)	.01	2.03 ± .12 (9)	2.28 ± .13 (12)	.02
J-111	3.45 ± .05 (9)	3.40 ± .52 (10)	.50	3.29 ± .14 (9)	3.16 ± .05 (10)	.30

* Probability (Student "t" Test)

† Standard Deviation. S.D. $= \sqrt{\frac{\Sigma(X-\bar{X})^2}{n-1}}$

§ Number of determinations.

Table III

Each culture dish was inoculated with 2×10^6 cells suspended in 1 ml of basic or drug medium.

Glucose disappearance is the difference between the glucose concentration at time zero and the glucose concentration in the dish after 4 hours of incubation. Lactate formed is the net increase in lactic acid found in the media after incubation. All figures represent averages of the indicated number of determinations. All values were analyzed for significance using the Student "t" Test and were considered significant at $P = .02$.

Table III

The effect of 6-MP on glucose disappearance and lactate formation in the media of HeLa, HEp-2, and J-111 cells sensitive and resistant to 6-MP.

	Glucose Disappearance (micromoles/ml)		P*	Lactate Formation (micromoles/ml)		P*
	Sensitive + 6-MP	Resistant + 6-MP		Sensitive + 6-MP	Resistant + 6-MP	
HeLa	3.23 ± .44† (10)§	1.40 ± .11 (11)	.001	2.87 ± .16 (10)	1.40 ± .11 (11)	.001
HEp-2	2.52 ± .17 (12)	1.98 ± .08 (10)	.001	2.28 ± .13 (12)	1.90 ± .10 (10)	.01
J-111	3.40 ± .52 (10)	3.23 ± .06 (8)	.30	3.16 ± .05 (10)	3.22 ± .16 (8)	.50

* Probability (Student "t" Test).

† Standard Deviation. S.D. $= \sqrt{\frac{\Sigma(X-\bar{X})^2}{n-1}}$

§ Number of determinations.

Discussion

The glucose, which disappeared from the cell culture media after incubation, was assumed to be taken up by the cells. It is assumed that the increased amount of glucose which disappeared after sensitive HeLa and HEp-2 cells were exposed to 6-MP was the result of the activity of the drug on the ability of the cells to increase the uptake of glucose. The increased amounts of lactate which appeared in the cell suspension media probably reflect the metabolism, by the cells, of the extracellular glucose or the breakdown of intracellular glycogen. In these experiments there is no way to distinguish which of these two sources contributed to the observed rise in lactate concentration.

The glycolytic activity of 6-MP in sensitive cells.

In my experiments it was found that of the three cell lines examined, only two (HeLa and HEp-2) were sensitive to the stimulation of glucose disappearance from their media by the activity of 6-MP. In one cell line, HeLa, the amount of glucose which disappeared from the media was independent of the concentration of 6-MP employed. The media of J-111 cells, on the other hand, was not depleted of glucose upon the addition of 6-MP. This latter finding does not agree with the observations of Katchman et al.(18) They reported that in the media of some 30 separate kinds of human cancer cells examined, 6-MP enhanced glucose disappearance.(18) J-111 cells therefore appear to be unique with respect to their inability to enhance the disappearance of glucose from their incubation media treated with 6-MP.

Also observed in my experiments was an increase in lactate

formation in the media of HeLa and HEp-2 cells treated with 6-MP. This finding suggests that not only does 6-MP stimulate glucose disappearance from the media, but it also enhances the aerobic production of lactic acid. These observations would seem to confirm similar findings of others.(19) Katchman et al., early investigations did not reveal a significant increase in lactate production associated with increased glucose disappearance;(18) however, later work demonstrated that lactate was increased by the activity of 6-MP.(19)

Katchman et al. have offered two mechanisms by which they explain the increased glycolytic activity brought about by addition of 6-MP, i.e., the enhancement of glucose disappearance and lactate formation by the action of 6-MP.(18,19) In their first publication they suggested that 6-MP stimulated the hexokinase reaction, hence the transport of glucose into the cell.(18) However, the work of Hochstein does not support this thesis.(15) Hochstein found that 6-MP has no effect on the hexokinase activity of the supernatant fraction of homogenized S91 mouse melanoma; indeed, hexokinase activity associated with the mitochondrial fraction was partially inhibited.(15)

In the most recent work of Katchman et al., the authors suggest that 6-MP may inhibit the Pasteur effect and thus allow aerobic glycolysis to proceed at an accelerated rate.(19) This proposal appears realistic in the light of recent evidence. Sulphydryl containing compounds, other than 6-MP, which have been shown to inhibit the Pasteur effect include: glutathione and cysteine.(30) A true Pasteur effect inhibitor has been defined as an agent which stimulates lac-

tate formation without inhibiting respiration,(29)

There is some evidence to suggest that 6-MP does not effect respiration at concentrations which enhance lactate formation, indicating 6-MP may indeed be acting as a Pasteur effect inhibitor.(8,19)

Other explanations for the glycolytic activity of 6-MP might include: 1) 6-MP may enhance respiration (oxygen uptake), hence the utilization of glucose, however, the evidence indicates that 6-MP has no effect on oxygen uptake;(8,19) 2) 6-MP may increase the levels of nicotinamide adenine dinucleotide (NAD) which, if present in rate limiting amounts, would stimulate glycolysis. Kaplan showed that the level of NAD is increased in mouse liver 20 hours after 6-MP administration.(17) My experiments were run only 4 hours; thus it is unlikely that NAD reached a level at which glycolysis was stimulated.

The principle result of the activity of 6-MP on sensitive cells is the inhibition of cell growth. Yet, my experiments and those of Katchman et al. have suggested that 6-MP may enhance glucose uptake and lactate formation, processes more compatible with viability than with growth-inhibition.(18,19) Therefore, one is tempted to speculate that the observed increase in glucose disappearance and lactate formation is the result of the cells' necessity to use glucose rather than the direct activity of 6-MP to stimulate glycolysis. Such a situation might arise if 6-MP inhibited the use or transport of nutrients, other than glucose. Under these conditions the cell could be required to compensate for the nutritional deficit and it might do so by enhancing the utilization of the readily available glucose. In this way 6-MP would appear to stimulate glycolytic activity, when in fact this drug

may only inhibit the cells' use of other vital non-carbohydrate nutrients. These proposals are more in keeping with the drug's principle activity, i.e., growth inhibition.

The glycolytic activity of 6-MP resistant cells.

Three HeLa cell sublines made resistant to 3 different concentrations of 6-MP were examined for their glycolytic activity. It was found that the stimulation of glucose disappearance by 6-MP, observed in the media of sensitive cells, did not occur in the media of 6-MP resistant cells. No significant differences in glucose disappearance were noted between the three cell sublines media. The media of one HeLa cell subline, resistant to 1.0 µg/ml 6-MP, was examined for the appearance of lactic acid (Table III). The data reveal that 6-MP does not enhance lactic acid formation in the media of HeLa or Hep-2 cells resistant to 1.0 µg/ml 6-MP. No significant alterations of glucose disappearance and lactate formation were observed in the media of J-111 cells resistant to 1.0 µg/ml 6-MP. Resistant HeLa, HEp-2, and J-111 cells resemble their untreated drug-sensitive cell counterparts in their glycolytic activity, in that namely the concentration of glucose and lactate in the media of each 6-MP resistant cell subline was in the same quantitative amounts as that found in the media of the corresponding drug-sensitive cell line. These experiments suggest that when HeLa and HEp-2 cells become resistant to 6-MP, they no longer are sensitive to glycolytic stimulation by 6-MP.

Brockman et al. have shown that the growth of HEp-2 cells is completely inhibited at 6-MP concentrations of 1.0 µg/ml.(6) An HEp-2 cell subline was developed resistant to 1.0 µg/ml 6-MP and was

found to grow in the presence of concentrations of 6-MP as high as 320 µg/ml.(6) These workers found that associated with 6-MP resistance by HEp-2 cells, the resistant cells lost their ability to form the 6-MP ribonucleotide. Indirect evidence suggests the action of 6-MP in 6-MP sensitive and resistant HeLa cells is identical to that described in HEp-2 cells.(12,13) The HEp-2 cells used in our experiments were sensitive to the growth-inhibitory effect of 6-MP at concentrations of 1.0 µg/ml. HEp-2 cells resistant to 6-MP were developed in a manner similar to the method described by Brockman et al. (6) Therefore, it is probable that the HEp-2 cell line and 6-MP resistant subline used in our experiments were demonstrating the same properties as those described in HEp-2 cells used by Brockman et al. (6)

My studies indicate that associated with 6-MP resistance in HeLa and HEp-2 cells, stimulation of glucose disappearance and lactate formation by the action of 6-MP is lost. The close parallelism between my cell preparations and those of Brockman et al. allows the suggestion that with 6-MP resistant HEp-2 and HeLa cells there may be a relationship between the resistant cells' inability to form the 6-MP ribonucleotide and their inability to respond to glycolytic stimulation by 6-MP. The relationship of these two observations could be made clearer if it were known which form of 6-MP (i.e., the free base or 6-MP ribonucleotide) was acting to enhance glucose disappearance and lactate production in the media of the drug-sensitive cell. Nevertheless, it is suggested that both HeLa and HEp-2 cells develop resistance by a common pathway and that as a result of re-

sistance both cell types lose their ability to increase glycolytic activity after addition of 6-MP.

In my experiments, the media of drug-sensitive acute leukemic J-111 cells does not show changes in glucose or lactate concentrations upon the addition of 6-MP. Furthermore, the media of J-111 cells does not show concentration differences in glucose or lactate concomitant with the development of 6-MP resistance. These findings suggest that the mechanism of action of 6-MP in J-111 cells may not be the same as that observed in HeLa and HEp-2 cells. Likewise, 6-MP resistance in J-111 cells may develop by mechanisms different from those described in HeLa and HEp-2 cells. More important, however, is the suggestion that 6-MP resistance in J-111 cells may develop by mechanisms different from those described in HeLa and HEp-2 cells.

Summary

HeLa cells were exposed to three concentrations (1.0, 1.5, 2.0 µg/ml) of 6-MP and it was found that 6-MP stimulated the disappearance of glucose from the incubation medium. No relationship was found between the amount of glucose which disappeared and the concentration of 6-MP employed. The media of three 6-MP resistant HeLa cell sublines (each resistant to a different concentration of 6-MP) were shown to be refractory to changes in glucose concentrations in the presence of 6-MP.

Glucose disappearance and lactate formation in the media of HeLa, HEp-2, and J-111 cells were compared in 6-MP-treated and untreated drug-sensitive cell lines. All cell lines and resistant sublines were exposed to 1.0 µg/ml 6-MP. Both glucose disappearance and lactate production were stimulated in the media of 6-MP-treated HeLa and HEp-2 cells. The media of J-111 cells exposed to 6-MP showed no increase in glucose disappearance or lactate formation. The data indicate that about one half of the glucose disappearing from the medium by the activity of 6-MP could be accounted for as lactate. It was postulated that the increased production of lactate was due to 6-MP acting as an inhibitor of the Pasteur effect within the cell.

Sublines of HeLa, HEp-2, and J-111 cells resistant to 6-MP were found to be refractory to glycolytic stimulation by 6-MP as measured by glucose disappearance and lactate formation in the media. These findings suggest that with the development of resistance to 6-MP,

there is a decreased sensitivity to the glycolytic stimulatory activity of 6-MP. It is suggested that the loss of pyrophosphorylase activity by 6-MP resistant HeLa and HEp-2 cells, as demonstrated by others, may be related to the decreased capacity of resistant cells to respond to the glycolytic stimulation of 6-MP.(5,6) However, it appears that resistance in HeLa and HEp-2 cells develops by a common pathway. With sensitive acute leukemic J-111 cells, the media of which showed no alterations of glucose or lactate concentration by 6-MP, it was suggested that resistance may develop by still another mechanism.

References

1. Atkinson, M. R., J. F. Jackson and R. K. Morton. Substrate Specificity and Inhibition of Nicotinamide Mononucleotide-Adenyl Transferase of Liver Nuclei: Possible Mechanism of Effect of 6-Mercaptopurine on Tumor Growth. Nature, 192, 946-948, 1961.

2. Atkinson, M. R., J. F. Jackson, R. K. Morton and A. W. Murray. Nicotinamide 6-Mercaptopurine Dinucleotide and Related Compounds: Potential Sources of 6-Mercaptopurine Nucleotide in Chemotherapy. Nature, 196, 35-36, 1962.

3. Baker, H. T. and L. L. Bennett. Inhibition by 6-Mercaptopurine Ribonucleotide of the Conversion of Inosinic Acid to Adenylic Acid in Cell Free Systems from Mercaptopurine-sensitive and Mercaptopurine-resistant Mammalian Cells. Biochim. Biophys. Acta, 80, 497-499, 1964.

4. Bennett, L. L., L. Simpson, J. Golden and T. L. Barker. The Primary Site of Inhibition by 6-Mercaptopurine on the Purine Biosynthetic Pathway in Some Tumors in Vivo. Cancer Res., 23, 1574-1580, 1963.

5. Brockman, R. W. Biochemical Aspects of Mercaptopurine Inhibition and Resistance. Cancer Res., 23, 1191-1201, 1963.

6. Brockman, R. W., G. G. Kelley, P. Stutts and V. Copeland. Biochemical Aspects of Resistance to 6-Mercaptopurine in Human Epidermoid Carcinoma Cells in Culture. Nature, 191, 469-471, 1961.

7. Davidson, J. D. and T. S. Winter. Purine Nucleotide Pyrophosphorylases in 6-Mercaptopurine-sensitive and -resistant Human Leukemias. Cancer Res., 24, 261-267, 1964.

8. Ebina, T. and M. Kurosu. Effects of Antitumor Compounds and Radiation on the Glycolysis and Respiration of Ascites-Tumor Cells. J. Nat. Cancer Inst., 20, 1023-1036, 1958.

9. Fodor, P. J., D. A. Clarke and O. Bodansky. Enzyme, Nitrogen, and DNA Concentrations in Sarcoma 180 in Mice Treated with 6-Mercaptopurine. Cancer Res., 21, 232-237, 1961.

10. Fodor, P. J., D. A. Clarke and O. Bodansky. Glycolysis by Sarcoma 180 of Mice Treated with 6-Mercaptopurine. I. The Role of Various Enzymes in Lactate Production. Cancer Res., 22, 1230-1238, 1962.

11. Gey, G. O., W. D. Coffman and M. T. Kubicek. Tissue Culture Studies of the Proliferative Capacity of Cervical Carcinoma and Normal Epithelium. Cancer Res., 12, 264-265, 1952.

12. Hakala, M. T. and C. A. Nichol. Studies on the Mode of Action of 6-Mercaptopurine and Its Ribonucleoside on Mammalian Cells in Culture. J. Biol. Chem., 234, 3224-3228, 1959.

13. Hakala, M. T. and C. A. Nichol. Prevention of the Growth-Inhibitory Effect of 6-Mercaptopurine by 4-Aminoimidazole-5-carboxamide. Biochim. Biophys. Acta, 80, 665-668, 1964.

14. Hitchings, G. H. and G. B. Elion. Chemical Suppression of the Immune Response. Pharmacol. Rev., 15, 365-405, 1963.

15. Hochstein, P. Synergistic Effects of 6-Mercaptopurine and Methotrexate on Anaerobic Glycolysis by Subcellular Fractions of S91 Mouse Melanoma. Proc. Am. Assoc. Cancer Res., 2, 214, 1957.

16. Hochstein, P. Glycolysis by Tumor Mitochondria and the Action of Insulin. Science, 125, 496-498, 1957.

17. Kaplan, N. O., A. Goldin, S. R. Humphreys, M. M. Ciotti and F. E. Stolzenbach. Pyridine Nucleotide Synthesis in the Mouse. J. Biol. Chem., 219, 287-298, 1956.

18. Katchman, B. J., R. E. Zipf and J.P.F. Murphy. Effect of 6-Mercaptopurine on Carbohydrate Metabolism of Human Cancer Cells. Nature, 195, 500, 1962.

19. Katchman, B. J., R. E. Zipf and J.P.F. Murphy. The Effect of Chemotherapeutic Agents upon the Metabolism of Intact Human Cancer Cells. An in Vitro Technic for Cell Sensitivity. Clin. Chem., 9, 511-529, 1963.

20. Laszlo, J., J. Stengle, K. Wright and D. Burk. Effects of Chemotherapeutic Agents on Metabolism of Human Acute Leukemia Cells in Vitro. Proc. Soc. Exp. Biol. Med., 97, 127-131, 1958.

21. McCollister, R. J., W. R. Gilbert and J. B. Wyngaarden. Pseudofeedback Inhibition of Purine Synthesis by 6-Mercaptopurine and Other Purine Analogues. J. Clin. Invest., 41, 1383, 1962.

22. Mihich, E., D. A. Clarke and F. S. Philips. Effects of 6-Mercaptopurine on Respiration, Anaerobic Glycolysis and Succino-Dehydrogenase Activity in Normal and Tumor Tissues. Proc. Soc. Exp. Biol. Med., 92, 758-762, 1956.

23. Montgomery, J. A., G. J. Dixon, E. A. Dulmage, H. J. Thomas, R. W. Brockman and H. E. Skipper. Inhibition of 6-Mercaptopurine-resistant Cancer Cells in Culture by Bis (Thioinosine)-5', 5'''-Phosphate. Nature, 199, 769-772, 1963.

24. O'Brien, D. and F. A. Ibbott. Laboratory Manual of Pediatric Micro- and Ultramicro-Biochemical Techniques. Third edition. New York, Harper and Row, 1962, pp. 134-137.

25. Osgood, E. E. and J. H. Brooke. Continuous Tissue Culture of Leukocytes from Human Leukemic Bloods by Application of "Gradient" Principles. Blood, 10, 1010-1022, 1955.

26. Puck, T. T., S. J. Cieciura and A. Robinson. Genetics of Somatic Mammalian Cells. III. Long-Term Cultivation of Euploid Cells from Human and Animal Subjects. J. Exp. Med., 108, 945-955, 1958.

27. Schwartz, R., J. Stack and W. Dameshek. Effect of 6-Mercaptopurine on Antibody Production. Proc. Soc. Exp. Biol. Med., 99, 164-167, 1958.

28. Schwartz, R., A. Eisner and W. Dameshek. The Effect of 6-Mercaptopurine on Immune Responses. Clin. Research, 7, 39, 1959.

29. Strickland, L. H. The Pasteur Effect in Normal Yeast and its Inhibition by Various Agents. Biochem. J., 64, 503-515, 1956.

30. Sumner, J. B. and K. Myrback. The Enzymes, Vol. II. New York, Academic Press, 1951, pp. 624.

31. Toolan, H. W. Transplantable Human Neoplasms Maintained in Cortisone-treated Laboratory Animals: H. S. #1; H. Ep. #1; H. Ep. #2; H. Ep. #3; and H. Emb. Rh. #1. Cancer Res., 14, 660-666, 1954.

32. Vital Statistics of the United States 1959. Washington, D.C., U.S. Department of Health, Education, and Welfare, 1959, pp. 6-15.

33. Whitehead, R. W. Trends in Chemotherapy of Cancer. Postgrad. Med., 23, 148-155, 1958.